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ORIGINAL RESEARCH

Expression of TIM3/VISTA checkpoints and the CD68 macrophage-associated marker correlates with anti-PD1/PDL1 resistance: implications of immunogram heterogeneity

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ABSTRACT

Although immunotherapies have achieved remarkable salutary effects among subgroups of advanced cancers, most patients do not respond. We comprehensively evaluated biomarkers associated with the "cancer-immunity cycle" in the pan-cancer setting in order to understand the immune landscape of metastatic malignancies as well as anti-PD-1/PD-L1 inhibitor resistance mechanisms. Interrogation of 51 markers of the cancer-immunity cycle was performed in 101 patients with diverse malignancies using a clinical-grade RNA sequencing assay. Overall, the immune phenotypes demonstrated overexpression of multiple checkpoints including VISTA (15.8% of 101 patients), PD-L2 (10.9%), TIM3 (9.9%), LAG3 (8.9%), PD-L1 (6.9%) and CTLA4 (3.0%). Additionally, aberrant expression of macrophage-associated markers (e.g. CD68 and CSF1R; 11-23%), metabolic immune escape markers (e.g. ADORA2A and IDO1; 9-16%) and T-cell priming markers (e.g. CD40, GITR, ICOS and OX40; 4-31%) were observed. Most tumors (87.1%, 88/101) expressed distinct immune portfolios, with a median of six theoretically actionable biomarkers (pharmacologically tractable by Food and Drug Administration approved agents [on- or off-label] or with agents in clinical development). Overexpression of TIM-3, VISTA and CD68 were significantly associated with shorter progression-free survival (PFS) after anti-PD-1/PD-L1-based therapies (among 39 treated patients) (all P < .01). In conclusion, cancer-immunity cycle biomarker evaluation was feasible in diverse solid tumors. High expression of alternative checkpoints TIM-3 and VISTA and of the macrophage-associated markers CD68 were associated with significantly worse PFS after anti-PD-1/PD-L1-based therapies. Most patients had distinct and complex immune expression profiles suggesting the need for customized combinations of immunotherapy.

Introduction

Immunotherapies, including vaccine therapy, chimeric antigen receptor (CAR) T-cell therapy and immune checkpoint inhibitors (ICIs) have evolved rapidly in recent years as a consequence of our accumulating understanding of tumor immunity[1] Among these, ICIs, such as monoclonal antibodies against cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1) or programmed death ligand-1 (PD-L1), are some of the most successful examples of drug development and, consequently, immune therapy has become standard of care in diverse malignancies.[2] Indeed, ICIs have been reported to exhibit anti-tumor effects against a wide range of tumor types, including both solid and hematological malignancies.[3–7]

However, ICIs are effective in only a portion of patients (response rates ranging from <5% to >40%, depending on the cancer type). Further, many patients exhibit serious side effects and some may also show accelerated disease progression (known as hyperprogression).[8] Thus, there is an unmet need to

understand the biomarkers associated with anti-tumor immune effects in individual patients. To date, several markers have been identified as predictors of response to ICIs including, but not limited to; (i) high PD-L1 expression or amplification,[9,10] (ii) microsatellite instability)- high (MSI-H)[11] or deficiency in mismatch repair genes,[12] (iv) presence of tumor infiltrating lymphocytes (TILs)[13] and (v) high tumor mutation burden (TMB).[14] However, even in the presence of the aforementioned biomarkers, not all patients demonstrate response to ICIs.[15–17]

To further enhance the anti-cancer effect with ICIs, deeper interrogation of the immune tumor milieu is warranted. This issue is also relevant to the many clinical trials combining ICIs with other therapeutic modalities, such as chemotherapy, molecularly targeted drugs, radiotherapy, or with various immune modulators.[18–20] Although there are signals of improved anti-tumor effect with some combination approaches,[21–24] in large, preliminary results suggest that optimization of combinations is still needed. For example, epacadostat, an IDO1 inhibitor,

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showed promising results in a phase II trial; however, a pivotal phase III trial in advanced melanoma failed to demonstrate superiority with the addition of epacadostat to pembrolizumab when compared to pembrolizumab alone (ECHO-301/Keynote-252 study).[22] Moreover, the clinical efficacy of combinations of ipilimumab plus nivolumab when compared to nivolumab alone in patients advanced melanoma appears similar (overall survival [OS] rate at 3 years of 58% in the ipilimumab-plus-nivolumab group vs. 52% in the nivolumab group).[24]

In the field of genomics, there are several remarkable successes with the use of targeted drugs. The most striking response rates occur when patients with specific genomic alterations are selected to receive cognate antagonists: imatinib for the treatment of chronic myelogenous leukemia harboring *BCR-ABL* fusion gene,[25] anti-EGFR therapies (e.g. gefitinib, erlotinib, osimertinib) for epidermal growth factor receptor (*EGFR*)-mutant lung cancer,[26] and crizotinib for anaplastic lymphoma kinase (*ALK*)-mutant lung cancer.[27] Hence the key to success is choosing the right patient for the right drug based on matched biomarkers.[28–32] These results have been confirmed by meta-analyses of large datasets, including meta-analyses of 346 published phase I trials showed that a personalized (biomarker matched) strategy using genomic biomarkers was associated with significantly higher response rates compared with a non-matched approach (response rate, 42% vs. 5%; $p < .001$)[30]

Although anti-CTLA-4 inhibitors and anti-PD-1 or anti-PD-L1 inhibitors already have remarkable effects in a subgroup of patients, many patients still do not respond to these drugs. To enhance patient selection, especially for combination therapies, comprehensive understanding of the cancer immunogram landscape is imperative.[33] Each step in the cancer-immunity cycle is regulated by various stimulatory and inhibitory factors that can potentially be targets of interest to optimize the anti-cancer immune effect (Supplemental Table 1). It is conceivable that, as is the case with matching therapy to genomic targets, matching immune modulators to the individual patient immune landscape may optimize salutary effects. Therefore, in order to facilitate rational clinical trial development, and better understand response and resistance, we comprehensively analyzed pan-cancer immunity markers.

Results

Patient characteristics

Among 101 patients with diverse malignancies, the median age was 57.1 years (range: 24.6–87.1 years), and 61.4% (62/101) were women. The most common diagnosis was gastrointestinal cancers (non-colorectal, 30.7% [31/101] and colorectal cancers, 20.8% [21/101]), followed by gynecologic cancers (15.8% [16/101]) (Table 1). Among 101 patients, 39 patients received anti-PD-1/PD-L1 based regimens.

A variety of patterns of RNA expression level for immune markers were seen in patients with cancer (N = 101)

A variety of immune response markers were evaluated (N = 51 markers) and RNA expression was ranked on a scale of 1 to

Table 1. Patient characteristics (N = 101).

Basic characteristics (N = 101)	N (%)
Age, median (range) (years)	57.1 (24.6–87.1)
Sex, N (%)	
Women	62 (61.4%)
Men	39 (38.6%)
Ethnicity, N (%)	
Caucasian	64 (63.4%)
Asian	16 (15.8%)
Hispanic	11 (10.9%)
African American	3 (3.0%)
Other	7 (6.9%)
Types of cancer diagnosis, N (%)	
Gastrointestinal, non-colorectal	31 (30.7%)
Gastrointestinal, colorectal	21 (20.8%)
Gynecologic	16 (15.8%)
Lung, non-small cell	7 (6.9%)
Breast	7 (6.9%)
Head, neck and thyroid	5 (5.0%)
Skin/melanoma	3 (3.0%)
Other *	11 (10.9%)

*Other: Includes patients with sarcoma (N = 4), adrenocortical carcinoma (N = 3), carcinoma of unknown primary (N = 2), prostate cancer (N = 1), and mesothelioma (N = 1).

100 and stratified into "Very high" (95–100), "High" (85–94), "Moderate" (50–84), "Low" (20–49), and "Very Low" (0–19) based on an internal reference population[34] Among checkpoint markers, high or very high RNA expression were most commonly observed in VISTA (15.8% [16/101 of patients]) followed by PD-L2 (10.9% [11/101]), TIM3 (9.9% [10/101]), LAG3 (8.9% [9/101]) and PD-L1 (6.9% [7/101]). High/very high RNA expression in macrophage-associated markers (some of which are myeloid suppressors) were most commonly seen in CSF1R (22.8% [23/101]), CCR2 (13.9% [14/101]) and CD163 (12.9% [13/101]). Among metabolic immune escape markers, RNA was highly expressed in CD39 (15.8% [16/101]), IDO1 (12.9% [13/101]) and ADORA2A (8.9% [9/101]). T-cell primed markers were most commonly expressed among OX40 ligand (30.7% [31/101]) followed by ICOS ligand (25.7% [26/101]), CD86 (18.8% [19/101]), CD80 (12.9% [13/101]), GZMB (12.9% [13/101]) and GITR (11.9% [12/101]). Several pro-inflammatory response markers were highly expressed in IL1B (34.7% [35/101]), DDX58 (32.7% [33/101]) and MX1 (24.8% [25/101]). Lastly, among tumor infiltrating lymphocyte markers, high/very high RNA expression were seen among CD4 (18.8% [19/101]), FOXP3 (17.8% [18/101]) and KLRD1 (11.9% [12/101]) (Figure 1, Supplemental Table 2 and Supplemental Figure 1).

Most patients had potentially actionable cancer-immunity markers (N = 101)

Overall, 52.5% (N = 53) of the 101 patients had at least one cancer-immunity cycle associated biomarker which was theoretically actionable by an FDA-approved agent (on- or off-label). Additionally, 46.5% (N = 47) had at least one cancer-immunity biomarker which was potentially targetable with an agent that is in clinical investigation (Figure 2 and Supplemental Table 1). Altogether, 99.0% (N = 100) of the patients had at least one theoretically actionable biomarker either with agents that are approved by the Food and Drug Administration (FDA) (on- or off-label) or with agents that are in clinical trials. The median number of actionable
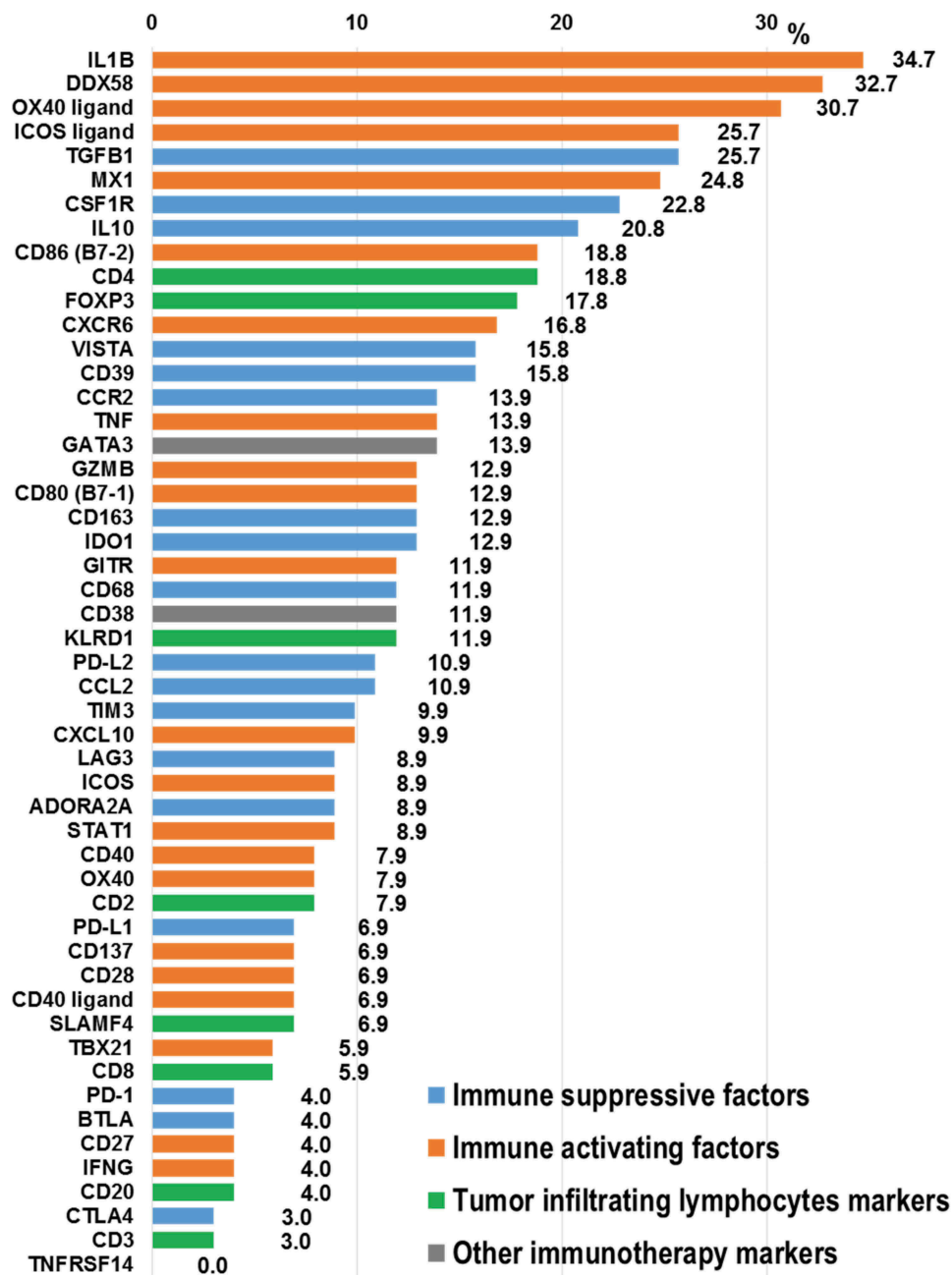
Figure 1. Frequency of high/very high RNA expression among cancer-immunity markers (N=101).

Among diverse cancer immunity markers evaluated, IL1B was most commonly highly expressed (34.7%) followed by DDX58 (32.7%), OX40 ligand (30.7%), ICOS ligand (25.7%) and TGFB1 (25.7%).

biomarkers that were associated with the cancer-immunity cycle was 6 (per patient) (range, 0–16).

Most patients had distinct expression patterns of cancer-immunity markers (N = 101)

Among the 101 patients, most patients (87.1% [88/101]) had different expression patterns of cancer-immunity markers (Figure 2). Four patients had high/very high RNA expression only in IL1B; three had high/very high RNA expression in both DDX58 and MX1; and six did not have highly expressed RNA in any of the cancer-immunity markers evaluated (Figure 2).

Patients with colorectal cancer had higher expression of IL-1B (inflammatory cytokine) than non-colorectal cancer

We examined the expression patterns of cancer-immunity markers between colorectal cancer (N = 21) and non-colorectal cancers (N = 80) and found that CCR2 (receptor for the CCL2; mediates signaling for chemotaxis) and GATA3 (highly expressed in helper T cells) were highly expressed among patients with non-colorectal cancers (high expression of CCR2 and GATA3 in colorectal vs. non-colorectal: 0% [0/21] vs. 17.5% [14/80], P = .038) (Supplemental Table 3). In contrast, high expression of IL1B was more commonly seen among patients with colorectal cancer (57.1% [12/21] vs. 28.7% [23/80], P = .021) (Supplemental Table 3). Moreover,

Table 2. Characteristics of patients who were treated with anti-PD-1/PD-L1 based immunotherapy (N = 39).

Basic characteristics	N (%)
Age, median (range) (years)	60.6 (31.6–87.1)
Sex, N (%)	
Women	23 (59.0%)
Men	16 (41.0%)
Type of cancer, N (%)	
Gastrointestinal, non-colorectal	11 (28.2%)
Gastrointestinal, colorectal	7 (17.9%)
Gynecologic	5 (12.8%)
Lung, non-small cell	5 (12.8%)
Head, neck and thyroid	3 (7.7%)
Skin/melanoma	3 (7.7%)
Breast	2 (5.1%)
Other *	3 (7.7%)
Anti-PD-1/PD-L1 based therapy administered as, N (%)	
First line	9 (23.1%)
Second line	11 (28.2%)
Third line	10 (25.6%)
≥ Fourth line	9 (23.1%)
Type of immunotherapy, N (%)	
Anti-PD-1/PD-L1 alone	13 (33.3%)
Anti-PD-1/PD-L1 with targeted agents	18 (46.2%)
Anti-PD-1/PD-L1 with chemotherapy	4 (10.3%)
Anti-PD-1/PD-L1 with anti-CTLA-4	3 (7.7%)
Anti-PD-1/PD-L1 with OX40 agonist	1 (2.6%)
Best response, N (%) (32 evaluable patients)	
Complete response	1 (2.6%)
Partial response	5 (12.8%)
Stable disease ≥ 6 months	8 (20.5%)
Stable disease < 6 months	1 (2.6%)
Progressive disease	17 (43.6%)
Response assessment unavailable or too early to be evaluated **	7 (17.9%)

*Other: Includes patients with sarcoma (N = 1); mesothelioma (N = 1); carcinoma of unknown primary (N = 1).

**N = 4 had stable disease at the time of data cutoff, however, follow up was less than 6 months and thus not included in the analysis. N = 3 without adequate clinical information to assess the response.

patients with gynecological cancers appear to have numerically higher frequencies of certain cancer-immunity markers when compared to other disease types including high expression of LAG3 (25%), GZMB (25%), IDO1 (31.3%), IL10 (43.8%) and OX40 ligand (62.5%) (though this did not reach statistical significance) (Supplemental Table 3).

High RNA expression in checkpoint markers TIM3 and VISTA as well as macrophage-associated markers CD68 as potential resistant markers for anti-PD-1/PD-L1 based regimens (N = 39)

Among 101 patients analyzed in this study, 39 received anti-PD-1/PD-L1 based regimens and were evaluated for progression-free survival (PFS) (Table 2). Among diverse cancer-immunity markers, high expression of TIM3 ($P = .007$), VISTA ($P = .001$), and CD68 ($P = .009$) were significantly associated with shorter PFS (median 1.7 versus 5.9 months in each case) (Table 3 and Supplemental Table 4) and were retained after the Bonferroni correction for multiple comparisons.

Among 39 patients who received anti-PD-1/PD-L1 based immunotherapy, 32 patients were evaluable for response. Clinical benefit was defined as achieving stable disease (SD) ≥ 6 months, partial response (PR) or complete response (CR). High expression of TIM3 and VISTA showed a trend toward lower rates of SD ≥6 months/PR/CR (0 versus 52% in each case; $p = .052$) as did CD68 (albeit weaker; 0 versus 50%;

$p = .113$) (Table 4). The small number of patients evaluable for response may have limited the analysis.

Discussion

Although immunotherapies, especially checkpoint inhibitors, have achieved some remarkable salutary anti-cancer effects among patients with numerous advanced malignancies, only a subgroup of patients respond to immunotherapies. Several biomarkers have been demonstrated to correlate with responsiveness.[8–12,14] However, as new immunotherapy agents have entered the clinical arena, a broader and more robust spectrum of predictive markers is needed, especially for combination treatments.[20,35] Herein, we comprehensively evaluated biomarkers associated with the "cancer-immunity cycle" among patients with diverse advanced solid tumors.

Patients appeared to have a wide variety of immune markers (Figure 2 and Supplemental Table 2). Along with the well-known checkpoint marker PD-L1, some tumors had high expression of other checkpoint markers (such as CTLA4 [3.0% of 101 patients had high expression], TIM3 [9.9%] and VISTA [15.8%]) as well as other factors involved in the cancer-immunity cycle including high expression of macrophage-associated markers (such as CD68 [11.9%], CCR2 [13.9%] and CSF1R [22.8%]), metabolic immune escape markers (such as ADORA2A [8.9%] and IDO1 [12.9%]), and anti-inflammatory immune markers (such as IL10 [20.8%]) (Figure 1, Supplemental Table 2 and Supplemental Figure 1). Theoretically, patients harboring high expression of immune-suppressive markers may benefit by targeting the specific inhibitory markers (e.g. to selectively administer IDO1 inhibitor among patients found to have high expression of IDO1) (Supplemental Table 1). On the other hand, expression of certain immune-stimulatory factors was low among patients with diverse cancers (e.g., very low/low expression was seen in ICOS ligand [38.6% of 101 patients]), CD40 ligand [53.5%] and GITR [53.5%]). For those patients with low expression of immune-stimulatory factors, it is conceivable that interventions that enhance the stimulatory factor may have a higher chance of demonstrating anti-cancer immune effects when compared to giving such stimulators to patients who may already have high endogenous expression of the molecule (Supplemental Table 1).

Especially pertinent for the development of various immunotherapy agents was the observation that the immune environment differed from patient to patient. Indeed, most patients (87.1%, 88/101) had distinct expression patterns of cancer-immunity markers. This observation is comparable to that reported in the cancer genomic field where the landscape of genomic alterations is complex and differs between patients even when they harbor tumors of the same histologic diagnosis[36] Admittedly, there are some pattern similarities within histologies (in both the genomic portfolios patients carry and, per our study, in the immune landscape of tumors). For instance, patients with colorectal cancer appeared to have higher expression of the inflammatory cytokine IL1B when compared to non-colorectal cancer patients (Supplemental Table 3). The high IL1B could conceivably be due to the fact that *KRAS* alterations (common in colorectal cancer) have
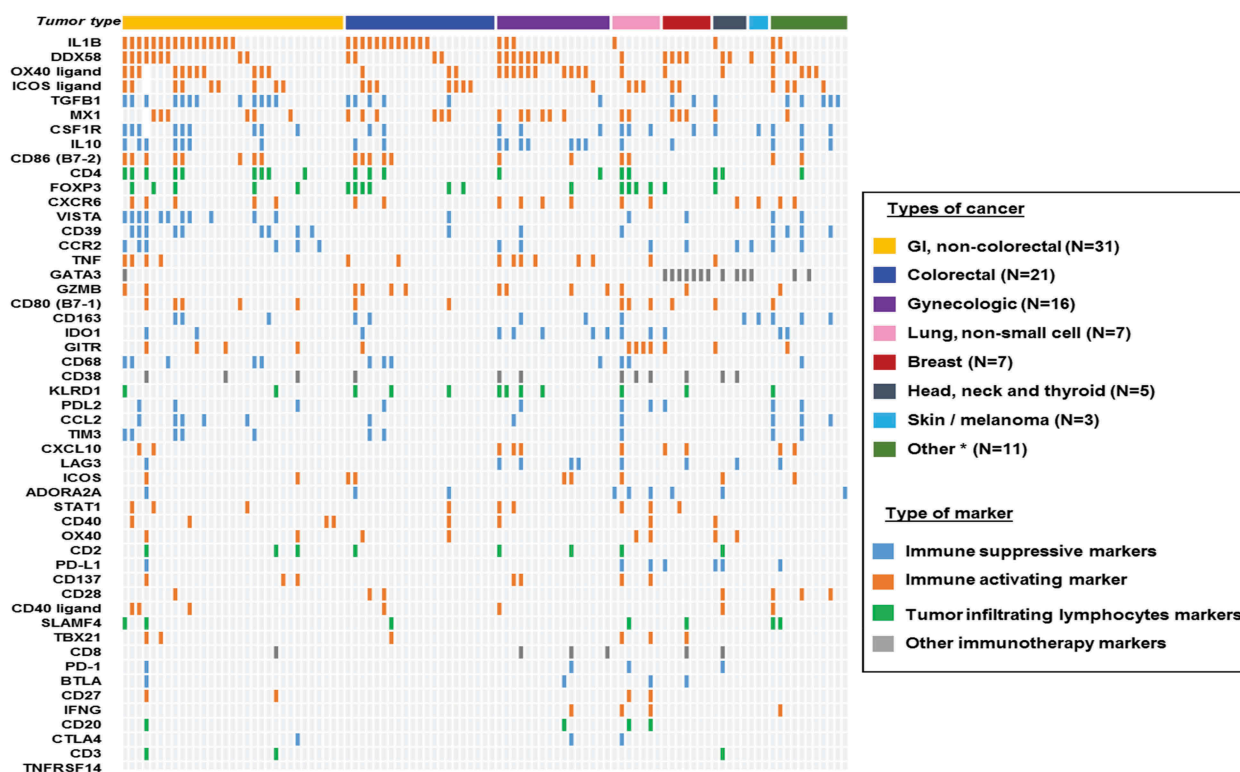
Figure 2. Overview of mRNA expression level of multiple immune markers for each individual cases (N = 101).
Among 101 patients evaluated for cancer-immunity markers, most patients (87.1% [88/101]) had a unique expression pattern of cancer-immunity markers.

been previously shown to stimulate signal transduction pathways that activate the IL1B promoter[37] Still, high IL1B was not exclusive to colorectal cancer, and an immunoprint of our patients (Figure 2) illustrates the diversity of immune portfolios within and between histologies. Additionally, most patients (99% [100/101]) had potentially targetable cancer-immunity markers that were pharmacologically tractable with either an FDA-approved agent (on- or off-label) or with an agent that is in clinical investigation (median number of potentially actionable immune biomarkers per patient: 6) (Figure 2 and Supplemental Table 1). Taken together, these findings suggest that the current standard of one-size-fits-all approach for immunotherapy may not be ideal for optimizing responsiveness and that obtaining an individualized immunogram [38] and treating on that basis warrants exploration.

Although durable clinical response can be seen with ICIs in a portion of patients, most patients do not demonstrate response or unfortunately have a short duration of clinical responses. Thus it is essential to understand the underlying biomarkers that may predict both response and resistance. In addition to known response markers (e.g. high TMB, MSI-high, deficiency in mismatch repair genes and highPD-L1 expression or amplification),[9–12,14] several resistance markers for immune therapy have also been reported: *PTEN, STK11/LKB1* alterations and activation of WNT/beta catenin for innate resistance markers, alterations in *JAK2* or *beta-2-microglobulin* as an acquired resistance, and *EGFR* and *MDM2* alterations as potential markers for hyperprogression.[8,39–42]

In this regard, we examined immune markers that might correlate with attenuated anti-PD-1/PD-L1 responsiveness

(Tables 3 and 4). Importantly, patients with high expression of alternative checkpoints such as TIM-3 and VISTA, as well as the macrophage-associated markers CD68, had significantly shorter PFS (Table 3). TIM-3, also known as Hepatitis A Virus Cellular Receptor 2 (HAVCR2), is a cell surface receptor expressed on activated T cells. Binding of C-type lectin galectin-9 (ligand of TIM-3) to TIM-3 leads to an immune inhibitory signal.[43] Further, TIM-3 upregulation is associated with resistance to anti-PD-1 therapies in preclinical models[44] Additionally, VISTA, also known as V-Set Immunoregulatory Receptor (VSIR), is an immunoregulatory receptor that inhibits the T-cell activation[45] Among patients with prostate cancer treated with ipilimumab (anti-CTLA-4 antibody), upregulation of VISTA was observed, suggesting a compensatory inhibitor pathway as a resistance mechanism after ipilimumab therapy[46] Moreover, increased expression of VISTA was seen among melanoma patients who progressed on anti-PD-1 inhibitor therapy, also suggesting a role for VISTA as an immune checkpoint[47] Since TIM-3 and VISTA were both implicated as resistant markers for anti-PD-1/PD-L1 based immunotherapy in our dataset, targeting of TIM-3 and VISTA may be required to achieve a better clinical outcome in selected patients. In the current study, high expression of CD68 was also significantly associated with poor PFS after anti-PD-1/PD-L1 based immunotherapy (Table 3). CD68 is a tumor-associated macrophage (TAM) marker that can have pro-tumoral and immunosuppressive functions. High TAM correlates with poor prognosis in breast cancer and myxoid liposarcoma patients.[48,49] In the preclinical setting, TAM has been reported to play a key role in resistance to

Table 3. Association between immune markers and progression-free survival among patients who received anti-PD-1/PD-L1 based regimens (N = 39).

Immune phenotypes	Incidence of High/Very high (%)	Median PFS time (Very high/High vs. Moderate/Low/Very low biomarker expression *) (Months)	P-value **
Checkpoint markers			
PD-1	1 (2.6%)	2.4 vs 4.4	0.423
PD-L1	3 (7.7%)	4.4 vs 2.7	0.335
PD-L2	6 (15.4%)	2.0 vs 5.9	0.229
Other Checkpoint markers			
BTLA	0 (0.0%)	- vs 4.4	-
CTLA-4	2 (5.1%)	4.4 vs 2.9	0.522
LAG3	3 (7.7%)	4.4 vs 2.9	0.638
TIM3	6 (15.4%)	1.7 vs 5.9	**0.007 *****
VISTA	5 (12.8%)	1.7 vs 5.9	**0.001 *****
TNFRSF14	0 (0.0%)	- vs 4.4	-
Macrophage-associated markers			
CCL2	8 (20.5%)	2.4 vs 5.9	0.096
CCR2	6 (15.4%)	1.7 vs 4.9	0.950
CD163	10 (25.6%)	2.7 vs 4.9	0.180
CD68	5 (12.8%)	1.7 vs 5.9	**0.009 *****
CSF1R	11 (28.2%)	2.4 vs 5.9	0.127
Metabolic immune escape markers			
ADORA2A	3 (7.7%)	- vs 2.9	0.188
CD39	7 (17.9%)	2.7 vs 5.9	0.176
IDO1	4 (10.3%)	4.4 vs 2.9	0.911
Anti-inflammatory response markers			
IL10	8 (20.5%)	2.0 vs 5.9	0.144
TGFB1	6 (15.4%)	1.7 vs 4.9	0.496
T-cell primed markers			
CD137	4 (10.3%)	4.4 vs 2.9	0.984
CD27	1 (2.6%)	2.4 vs 4.4	0.423
CD28	4 (10.3%)	1.7 vs 4.9	**0.018**
CD40	2 (5.1%)	2.0 vs 4.4	0.726
CD40 ligand	1 (2.6%)	2.0 vs 4.4	0.271
GITR	6 (15.4%)	15.5 vs 2.9	0.268
ICOS	3 (7.7%)	- vs 2.7	0.320
ICOS ligand	9 (23.1%)	2.7 vs 4.4	0.765
OX40	3 (7.7%)	20.6 vs 2.7	0.092
OX40 ligand	9 (23.1%)	2.4 vs 5.9	0.061
GZMB	5 (12.8%)	4.4 vs 2.9	0.740
IFNG	2 (5.1%)	4.4 vs 2.9	0.748
CD80 (B7-1)	7 (17.9%)	4.4 vs 2.9	0.638
CD86 (B7-2)	8 (20.5%)	2.0 vs 5.9	**0.030**
TBX21	2 (5.1%)	0.8 vs 4.9	0.156
Pro-inflammatory response markers			
IL1B	15 (38.5%)	2.7 vs 4.9	0.884
STAT1	3 (15.4%)	6.3 vs 2.7	0.963
TNF	5 (12.8%)	1.1 vs 4.4	0.337
DDX58	12 (30.8%)	4.4 vs 2.9	0.686
MX1	8 (20.5%)	4.4 vs 2.9	0.581
CXCL10	4 (10.3%)	4.4 vs 2.9	0.814
CXCR6	6 (15.4%)	4.4 vs 2.9	0.864
Tumor infiltrating lymphocytes markers			
CD2	3 (15.4%)	NR vs 2.7	0.320
CD3	0 (0.0%)	- vs 4.4	-
CD4	9 (23.1%)	2.4 vs 5.9	0.141
CD8	2 (5.1%)	0.9 vs 4.9	**0.009**
FOXP3	8 (20.5%)	4.4 vs 2.9	0.449
KLRD1	5 (12.8%)	2.0 vs 4.9	0.142
SLAMF4	4 (10.3%)	2.0 vs 4.9	0.257
CD20	1 (2.6%)	2.4 vs 4.4	0.423
Other immunotherapy markers			
CD38	6 (15.4%)	4.4 vs 2.9	0.605
GATA3	5 (12.8%)	5.9 vs 2.9	0.503

Abbreviation: HR, hazard ratio; PFS, progression-free survival; NR, not reached. All patient received anti-PD-1/PD-L1 based therapy (alone or in combination with other agent).

*See Methods for definition of very high, high, moderate, low, very low biomarker expression.

**P-values with univariate analysis by log-rank test.

***P-values significant after the Bonferroni correction. Cutoff for significant P-values were defined as 0.05/number of markers at each section. For example, there were 6 variables in "Other Checkpoint markers". For this category, significant P-values were defined as less than or equal to 0.0083 (0.05/6).

Table 4. Correlation between immune markers and clinical benefit (SD≥6m/PR/CR) from anti-PD-1/PD-L1 based immunotherapy (N = 32).

Immune markers	SD≥6m/PR/CR (%)		
	High/very high *	Moderate/low/very low *	P-value **
Checkpoint markers			
PD-1	0/1 (0.0%)	14/31 (45.2%)	> 0.999
PD-L1	1/1 (100%)	13/31 (41.9%)	0.438
PD-L2	1/5 (20.0%)	13/27 (48.1%)	0.355
Other Checkpoint markers			
BTLA	0/0 (0.0%)	14/32 (43.8%)	-
CTLA-4	1/1 (100%)	13/31 (41.9%)	0.438
LAG3	0/1 (0.0%)	14/31 (45.2%)	> 0.999
TIM3	0/5 (0.0%)	14/27 (51.9%)	**0.052**
VISTA	0/5 (0.0%)	14/27 (51.9%)	**0.052**
TNFRSF14	0/0 (0.0%)	14/32 (43.8%)	-
Macrophage-associated markers			
CCL2	2/7 (28.6%)	12/25 (48.0%)	0.426
CCR2	2/6 (33.3%)	12/26 (46.2%)	0.672
CD163	1/6 (16.7%)	13/26 (50.0%)	0.196
CD68	0/4 (0.0%)	14/28 (50.0%)	0.113
CSF1R	3/10 (30.0%)	11/22 (50.0%)	0.446
Metabolic immune escape markers			
ADORA2A	1/2 (50.0%)	13/30 (43.3%)	> 0.999
CD39	1/5 (20.0%)	13/27 (48.1%)	0.355
IDO1	1/2 (50.0%)	13/30 (43.3%)	> 0.999
Anti-inflammatory response markers			
IL10	0/5 (0.0%)	14/27 (51.9%)	0.052
TGFB1	1/5 (20.0%)	13/27 (48.1%)	0.355
T-cell primed markers			
CD137	2/3 (66.7%)	12/29 (41.4%)	0.568
CD27	0/1 (0.0%)	14/31 (45.2%)	> 0.999
CD28	0/4 (0.0%)	14/28 (50.0%)	0.113
CD40	1/2 (50.0%)	13/30 (43.3%)	> 0.999
CD40 ligand	0/1 (0.0%)	14/31 (45.2%)	> 0.999
GITR	5/6 (83.3%)	9/26 (34.6%)	0.064
ICOS	1/1 (100%)	13/31 (41.9%)	0.438
ICOS ligand	3/8 (37.5%)	11/24 (45.8%)	> 0.999
OX40	3/3 (100%)	11/29 (37.9%)	0.073
OX40 ligand	1/7 (14.3%)	13/25 (52.0%)	0.104
GZMB	0/2 (0.0%)	14/30 (46.7%)	0.492
IFNG	0/0 (0.0%)	14/32 (43.8%)	-
CD80 (B7-1)	2/5 (40.0%)	12/27 (44.4%)	> 0.999
CD86 (B7-2)	0/6 (0.0%)	14/26 (53.8%)	**0.024**
TBX21	0/1 (0.0%)	14/31 (45.2%)	> 0.999
Pro-inflammatory response markers			
IL1B	5/13 (38.5%)	9/19 (47.4%)	0.725
STAT1	2/2 (100%)	12/30 (40.0%)	0.183
TNF	2/5 (40.0%)	12/27 (44.4%)	> 0.999
DDX58	4/10 (40.0%)	10/22 (45.5%)	> 0.999
MX1	3/7 (42.9%)	11/25 (44.0%)	> 0.999
CXCL10	1/2 (50.0%)	13/30 (43.3%)	> 0.999
CXCR6	1/3 (33.3%)	13/29 (44.8%)	> 0.999
Tumor infiltrating lymphocytes markers			
CD2	1/1 (100%)	13/31 (41.9%)	0.438
CD3	0/0 (0.0%)	14/32 (43.8%)	-
CD4	1/7 (14.3%)	13/25 (52.0%)	0.104
CD8	0/2 (0.0%)	14/30 (46.7%)	0.492
FOXP3	3/6 (50.0%)	11/26 (42.3%)	> 0.999
KLRD1	0/3 (0.0%)	14/29 (48.3%)	0.238
SLAMF4	0/3 (0.0%)	14/29 (48.3%)	0.238
CD20	0/1 (0.0%)	14/31 (45.2%)	> 0.999
Other immunotherapy markers			
CD38	3/4 (75.0%)	11/28 (39.3%)	0.295
GATA3	2/4 (50.0%)	12/28 (42.9%)	> 0.999

*See Methods for definition of very high, high, moderate, low, very low biomarker expression.

**P-values with univariate analysis by Fisher's exact test; no P-values were statistically significant after the Bonferroni correction; cutoff for significant P-values were defined as 0.05/number of markers in each section.

Abbreviation: CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease

anti-PD-1 therapy,[50] which is in line with our current clinical observations.

There were several limitations to our study. First, the sample size was relatively small and was heterogeneous. Thus, further

validation in a larger dataset is needed. This is especially pertinent for the clinical data, which requires a larger sample size that would permit a multivariate analysis. Second, since the number of patients for each cancer diagnosis was based on the number of tests requested for cancer-immunity cycle markers by the treating physician, there is a potential of sample size bias. Third, though the cancer immune landscape data was collected prospectively, the analysis of correlation with immunotherapy treatment outcome was evaluated retrospectively. Fourth, mRNA expression does not always correlate the protein expression. Moreover, immune markers can be expressed on both immune and tumor cells, which may be differentiated by immunohistochemistry testing, and requires further investigation. Fifth, dynamic changes in cancer-immunity markers can be observed, especially with anti-cancer therapies. In-depth analysis of the association between therapy and immunity markers is required. Despite these limitations, the current report provides a comprehensive analysis of cancer-immunity cycle markers and clinical correlates in the pan-cancer setting.

In conclusion, we have investigated 101 patients with diverse cancers and demonstrated that most patients (87.1%, 88/101) had distinct (and complex) patterns of cancer-immunity markers. The majority of patients (99% [100/101]) had multiple potentially targetable cancer-immunity markers (with either an FDA-approved agent [on- or off-label] or with an agent that is in clinical investigation). Therapeutically, high expression of the checkpoints TIM-3 and VISTA as well as the macrophage-associated marker CD68 were associated with significantly worse PFS after anti-PD-1/PD-L1 based immunotherapies. Our observations suggest that upregulation of alternative checkpoints or myeloid suppression (via macrophage-associated markers) may diminish responsiveness to anti-PD-1/PD-L1 agents. Furthermore, individualizing immunotherapy agents to each patient's tumor immune portfolio merits investigation for optimized outcomes.

Materials and methods

Patients

The cancer-immunity markers among 101 eligible consecutive patients with diverse solid cancers seen at the University of California San Diego Moores Cancer Center for Personalized Therapy were examined at a Clinical Laboratory Improvement Amendments (CLIA)-licensed and College of American Pathologist (CAP)-accredited clinical laboratory, OmniSeq (https://www.omniseq.com/). We used the electronic medical record to curate the clinical characteristics of these patients. All investigations followed the guidelines of the UCSD Institutional Review Board for data collection (Profile Related Evidence Determining Individualized Cancer Therapy, NCT02478931) and for any investigational therapies for which the patients consented. Among patients who had anti-PD-1/PD-L1 based therapy (N = 39), all samples were collected prior to the checkpoint inhibitor based therapy.

Tissue samples and analysis of cancer-immunity markers

Tumors were provided as formalin-fixed, paraffin-embedded (FFPE) samples and evaluated with RNA sequencing by

OmniSeq laboratory. Briefly, total RNA was extracted from FFPE by means of the truXTRAC FFPE extraction kit (Covaris, Inc., Woburn, MA), following the manufacturer's instructions with some modifications. Following purification, RNA was eluted in 50 μL water and yield was determined by the Quant-iT RNA HS Assay (Thermo Fisher Scientific, Waltham, MA), as per manufacturer's recommendation. A predefined yield of 10 ng RNA was used as acceptance criteria to ensure adequate library preparation. RNA-sequencing absolute reads were generated using Torrent Suite's plugin immuneResponseRNA (v5.2.0.0)[34] The RNA library was prepared to measure RNA expression of 51 targeted immune response markers including checkpoint markers (N = 9 markers including PD-L1, PD-L2, CTLA-4 and LAG3), macrophage-associated markers (N = 5 markers including CCR2, CD68 and CSF1R), metabolic immune escape markers (N = 3 markers including ADORA2A and IDO1), anti-inflammatory response markers (N = 2 markers, IL10 and TGFB1), T-cell primed markers (N = 15 markers including CD40, GITR, ICOS and OX40), pro-inflammatory response markers (N = 7 markers including IL1B and TNF), tumor infiltrating lymphocytes markers (N = 8 markers including CD4, CD8 and FOXP3) and other immunotherapy markers (N = 2) (Supplemental Table 1). Transcript abundance was normalized and compared to an internal reference population (N = 735 patients with diverse cancers) which were used to rank RNA expression in test samples[34] Rank values were set on a scale of 1 to 100 and stratified into "Very high" (95–100), "High" (85–94), "Moderate" (50–84), "Low" (20–49), and "Very Low" (0–19). Tissue samples were obtained via biopsies in 54 patients (53.5%) and via surgically resected samples in 47 patients (46.5%). Moreover, in 50 patients (49.5%), the biopsies were from the primary tumor and in 49 patients (48.5%) they were from metastatic sites (N = 2 with carcinoma of unknown primary).

Endpoints and statistical methods

Patient characteristics and the pattern of cancer-immunity markers were summarized by descriptive statistics. The Fisher's exact test and the Bonferroni correction were used for categorical variables. Response to anti-PD-1/PD-L1 based immunotherapy were assessed by imaging (e.g. computed tomography and/or magnetic resonance imaging) and categorized into progressive disease, SD, PR and CR according to the treating physician's evaluation (immunotherapy response is evaluated by iRECIST criteria). PFS was defined as time interval between the start of therapy and the date of disease progression. Patients with ongoing therapy without progression at the last follow up date were censored for PFS at that date. Log-rank test and Bonferroni correction were used to compare subgroups of patients. All tests were 2-sided and P-values ≤ 0.05 were considered significant. Statistical analyses were performed with assistance from coauthor RO using SPSS version 24.0 (Chicago, IL, USA).

Disclosure of Potential Conflicts of Interest

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